# Supporting Information

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## SI Text

**Preparation of 7,8-DHF.** Several commercially available 7,8-DHF preparations were analyzed and found to contain between 2% and 10% folic acid and up to 1% THF, often in an inverse relationship. Material produced by Blakley's modification (1) of the Futterman procedure (2) was found to initially contain 0.8% folic acid and 1% THF. The latter impurity could be decreased by not quite 2-fold by subsequent acid precipitation as described by Blakley (1). However, several aspects of the Futterman method make this difficult to reproduce with uniform results, especially lack of pH control. Therefore, the reduction of folic acid with sodium dithionite has been further optimized, and an efficient purification capable of removing trace THF developed.

7,8-DHF calcium salt was prepared by dissolving 73 mg (0.15 mmol) folic acid· $2.1H_2O$ (98%) (Sigma 7876) in 5.0 mL of 1 M potassium phosphate buffer pH 7.45, placed in a bath at 23 °C, sparged thoroughly with argon, and 0.26 g 87% sodium dithionite (Fluke 71699) added. The mixture was continuously bubbled with argon for 15 min (by which time the pH had decreased to between 6.9 and 7.0) and then added to 20 ml of ice cold argon sparged water. Starting at 20 min, with the mixture still on ice, about 48 ml of deaerated 0.2 M ascorbic acid was added at 0.8 ml/min using a Harvard infusion pump, with continuous vigorous bubbling of inert gas until pH 4.0 was reached. The mixture was kept for an additional hour on ice to permit completion of precipitation, centrifuged under argon, the precipitate washed with 10 ml of 0.1 M ascorbic acid, and again centrifuged. A small sample of the off-white precipitate was dissolved in 10 mM sodium ascorbate and analyzed by HPLC on a 3$\mu$ Luna phenylhexyl (Phenomenex) 15 × 0.46 cm column eluted at 1.0 ml/min with 40 mM $K_2HPO_4$ plus $H_3PO_4$ to pH 7.0, 1 mM EDTA/acetonitrile (98:2) and monitored with a Waters 996 photodiode array detector. Greater than 98% of the chromatogram at 282 nm (excluding the peak due to ascorbic acid) was associated with 7,8-DHF. THF was typically present in the range of 0.3–0.4%, and unreduced folic acid between 0% and 0.3%.

To the precipitate was added 8 ml of deaerated water, the suspension further sparged with argon, placed in a 45 °C bath, put into solution by addition of 1 M NaOH (to approximately pH 6.2), and 0.45 ml 1 M $CaCl_2$ added. This was allowed to passively cool in a refrigerator at 3 °C for 30 to 60 min, a small first crop collected, washed once with 10 ml of 10 mM ascorbic acid, and dried under vacuum over $P_2O_5$ to give 10.2 mg (which includes some residual ascorbic acid). The supernatant was further cooled on ice for 1 to 2 hours and a second crop collected, similarly washed and dried to give 42 mg pale tan powder (absorbance ratio 340/281 nm: 0.25). Any unreduced folic acid precipitates early with the first crop, whereas THF remains in the final supernatant. Extended cooling of the crystallization mixture in an attempt to recover all product can eventually also precipitate THF. HPLC analysis showed that both the first and second crops contained less than 0.1% THF and the second crop undetectable ($< 0.1\%$) folic acid. The presence of residual ascorbic acid helps to maintain the product, which is stable if kept under inert gas at −20 °C.

The reduction of folic acid to 7,8-DHF, as well as the subsequent reduction to THF, takes place in 2 steps: the formation of a dithionite adduct (hypothesized to be a sulfinate) followed by displacement by acid (3). Analysis by HPLC of the reaction described by Blakley (1) after dithionite addition, but before acidification, revealed that although folic acid had been mostly consumed, the mixture also contained a pair of early eluting compounds both having spectra similar to the adduct described by Scrimgeour as well as 7,8-DHF. The slow addition of acid, therefore, also assists in completion of the reaction as well as promoting product precipitation. The overall shape of the chromatogram suggested that the pair of peaks (possibly diastereoisomers) were undergoing on-column conversion to product. The rate of addition of bisulfite (and presumably also dithionite) to 7,8-DHF is several orders-of-magnitude faster than with folic acid. Thus, the low amount of THF formed despite considerable excess of dithionite appears to be due, in part, to the slow rate of final decomposition of the dihydrofolate adduct, especially in reactions that are not heated. However, it was also found that use of minimal dithionite (about 8 mol/mol), and dilution into cold water helped to decrease the contamination with THF. Accurate timing of this dilution was facilitated by buffering initially to pH 7.45, which slows the reaction in comparison to that described by Futterman or Blakley (1, 2) (carried out in the vicinity of pH 6). The higher pH also helped to control the rate of formation of THF.

**DHFR Assay Conditions.** One consequence of the inhibition of DHFR by folic acid (noncompetitive at low 7,8-DHF, but competitive at high concentrations) is that the rate obtained with 7,8-DHF is dependent on the purity of this substrate. For example, we found that even freshly prepared 7,8-DHF from a major supplier produced a rate nearly 3 times lower than with material prepared as described here. This could be entirely ascribed to contamination with folic acid.

The tetrahydrofolic acid product of the reaction is highly unstable. While this can be adequately maintained during the reaction by the presence of mercaptoethanol, the addition of TCA to precipitate protein defeats the reducing activity of the mercaptoethanol which is most effective as the thiolate anion. Thus, 0.1 M ascorbate is added with the TCA, and while this also is not optimal at acidic pH, this none the less allows recovery of >95% of the THF subsequent to the 10 min centrifugation. Rapid neutralization of the resulting supernatant is important for consistent recovery. A pH between 4.0 and 4.5 is a compromise between both having ascorbate anion present and also putting a positive charge on N5 of the THF which minimizes autooxidation. The slight loss of THF during this process is compensated by parallel treatment of the standards. Further, the validity of the assay procedures is indicated by the agreement between the spectrophotometric and HPLC assays (see Table 1).

A concentration of 0.1 mM NADPH was used to saturate both the rat and human enzymes. Literature values for the $K_m$ of NADPH with 7,8-DHF as substrate range between 0.16 to 5.9 $\mu$M with the human enzyme at neutral pH. This spread can be easily rationalized by the report of a biphasic response to NADPH with 2 $K_m$'s (0.16 and 4.2 $\mu$M) that results from a partially ordered bi-bi reaction mechanism (4). For the rat enzyme, a $K_m$ of 0.72 $\mu$M at pH 6.5 has been reported (5). Since the rate of conversion of the central ternary complex is extremely fast with 7,8-DHF as substrate, $K_m$ for both reactants is largely determined by their association rates and the off rates of the products. With folic acid as saturating substrate, the chemical conversion becomes so much slower (see Table 2) that the $K_m$ for NADPH is driven close to its $K_d$ for the E-folate complex. The values for the on and off rates (6) indicate that the $K_d$ for NADPH is substantially less than 1 $\mu$M. Thus, the $K_m$ for NADPH with folic acid as substrate is even lower than its $K_m$ with 7,8-DHF as substrate.

**Calculation of DHFR Rate in Human Liver with Folic Acid as Substrate.** The activity at saturating 7,8-DHF ranged between 5.4 and 26 nmol/min/g wet weight liver at 27 °C for the 6 subjects we examined (Fig. 2*A*). However, since we also show that folic acid results in a rate at $V_{max}$ that is 1300 times slower than with the natural substrate (Table 3), this translates to the reduction of 7.5–36 nmol/min of FA for the entirety of a 1.8-kg liver. Finally, the rate with rat liver DHFR and folic acid is substrate at 37 °C was found to be 2.1 times faster than at 27 °C (1.3 vs. 0.60 nmol THF/min/g wet weight liver, respectively, from 16 pooled Wistar rat livers; see Table 2). Assuming this temperature dependence of rates applies to human DHFR, a 1.8-kg human liver would have the capacity to reduce 16–75 nmol FA/min.


1. Blakley RL (1960) Crystalline dihydropteroylglutamic acid. *Nature* 188:231–232.
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6. Tsay JT, et al. (1990) Kinetic investigation of the functional role of phenylalanine-31 of recombinant human dihydrofolate reductase. *Biochemistry* 29:6428–6436.

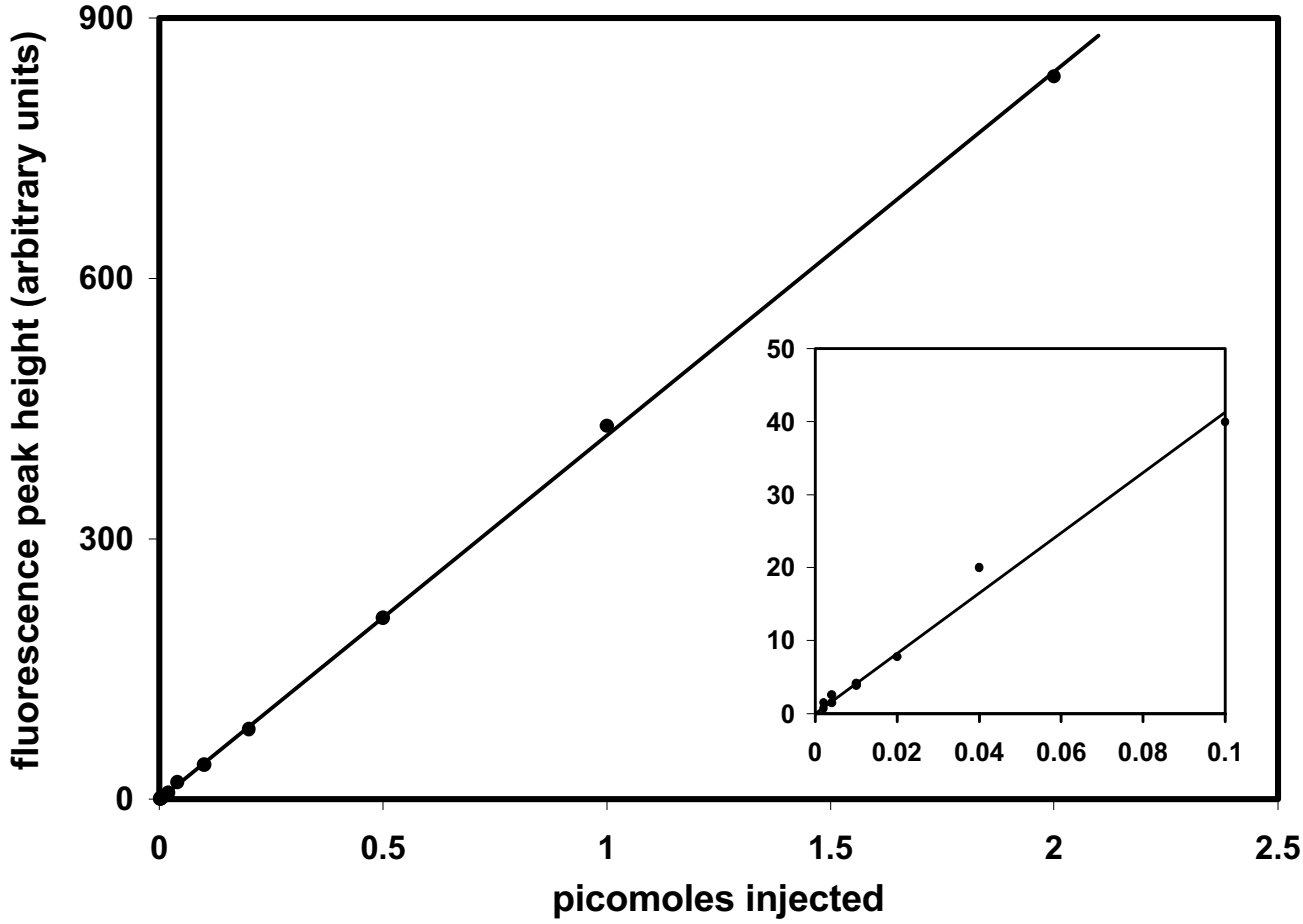


**Fig. S1.** The linearity of fluorescence response to THF standards up to 2.5 picomoles injected in a volume of 20 $\mu$L. The *Inset* expands the lower part of the data up to 0.1 picomoles. This degree of linearity, especially at the lower end of the range, is highly dependent on the presence of DTT in the mobile phase.

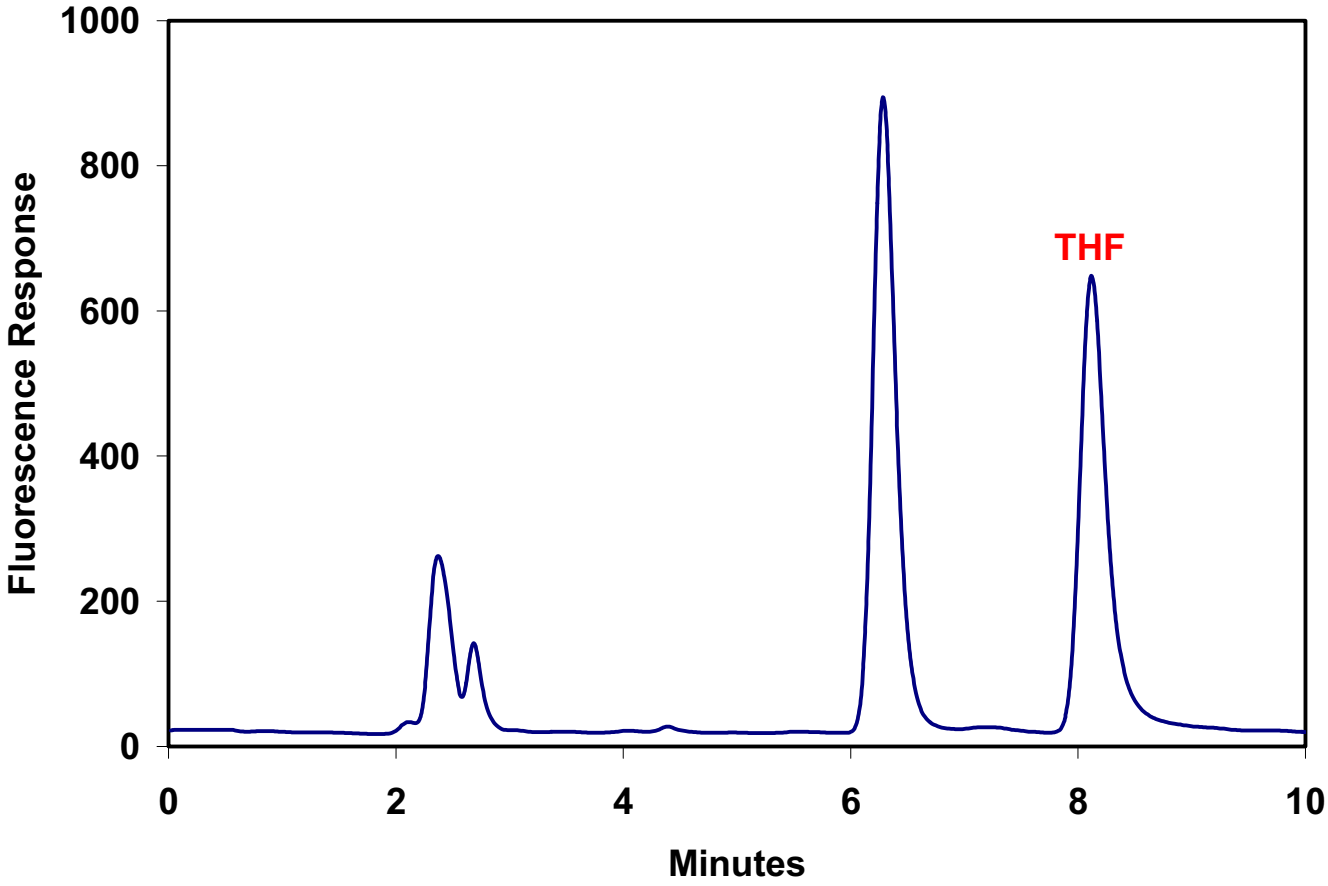


**Fig. S2.** Chromatogram of 20 $\mu$L from a DHFR reaction with 40 $\mu$L of 100,000 $\times$ *g* supernatant (1 g liver:3 mL homogenizing buffer) of human liver from subject number 3 in a 0.5-mL reaction.

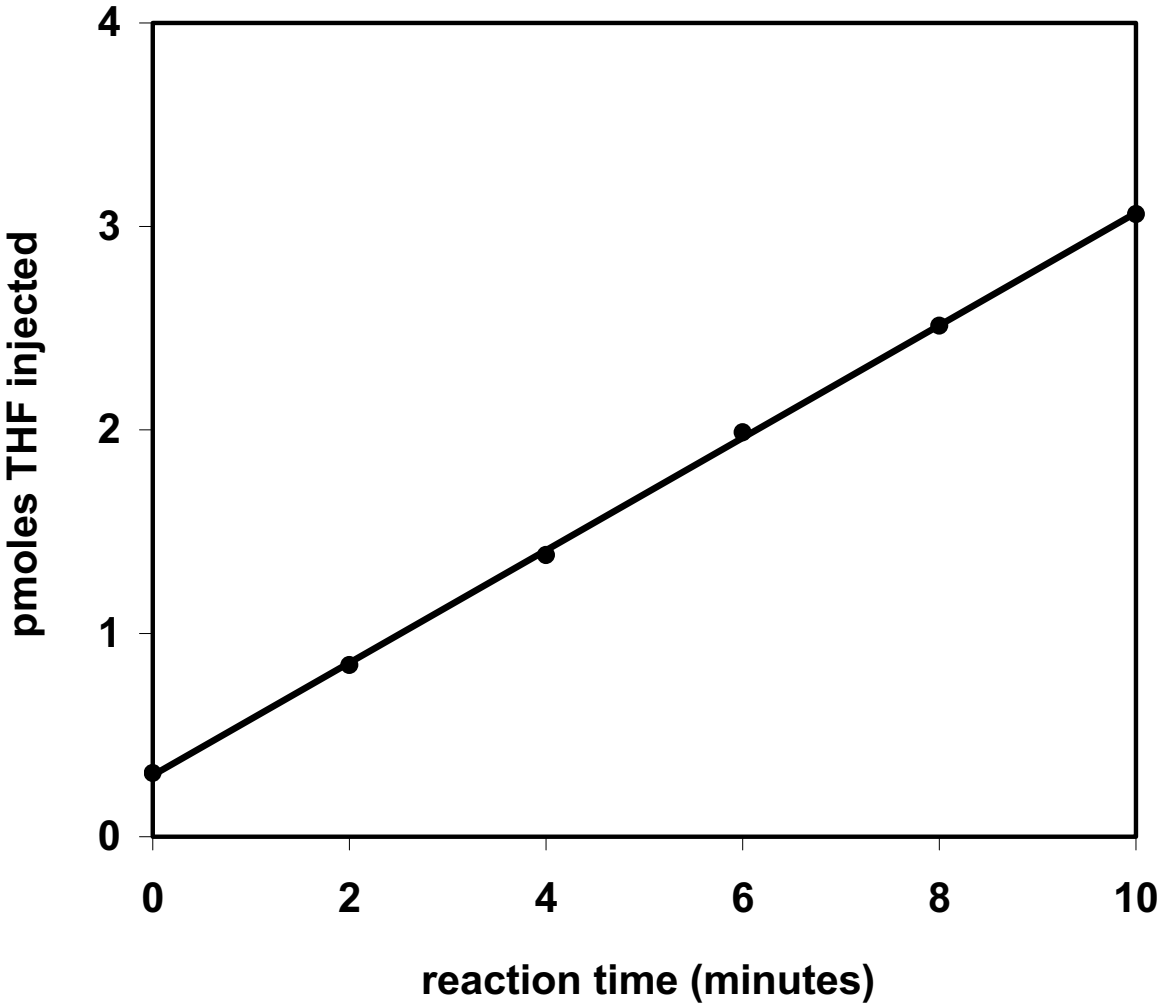


**Fig. S3.** DHFR activity as a function of time. The reaction contained 30 $\mu$L of human liver extract from subject 6 in a total volume of 0.5 mL.

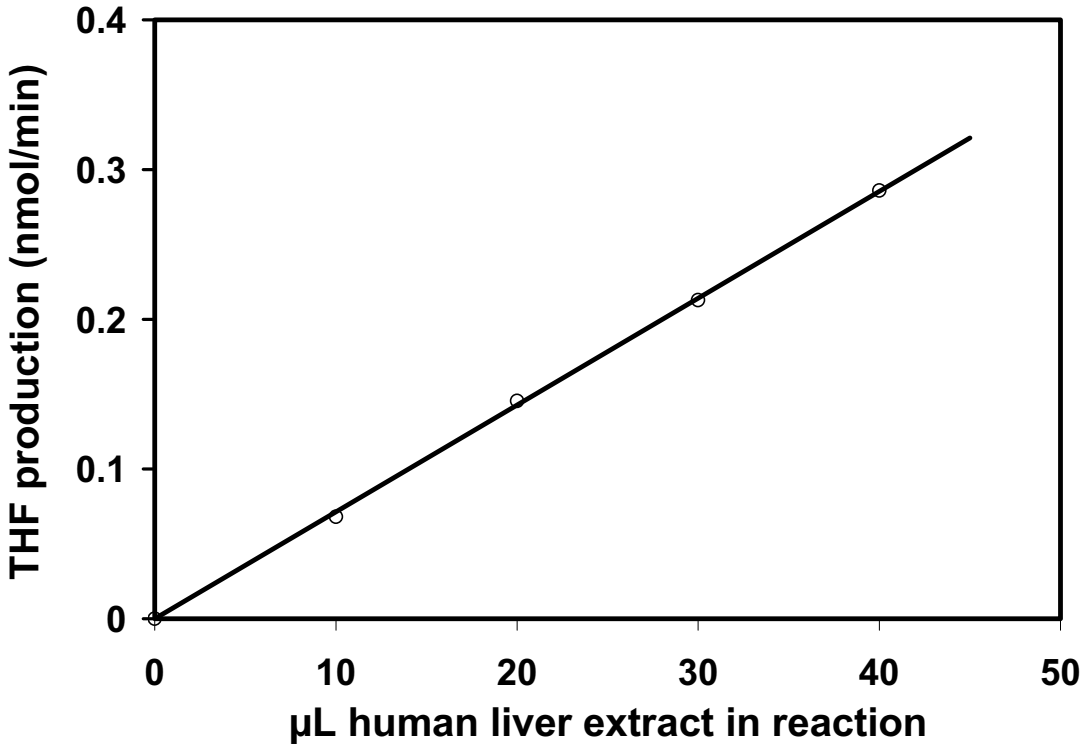


**Fig. S4.** DHFR activity as a function of the volume of a crude extract of human liver included in a 0.5 mL total reaction volume. The extract is the 100,000 × *g* supernatant of a homogenate from subject number 6. The rates are derived from the slopes from the best fits to each time course, one of which is shown in Fig. S3.

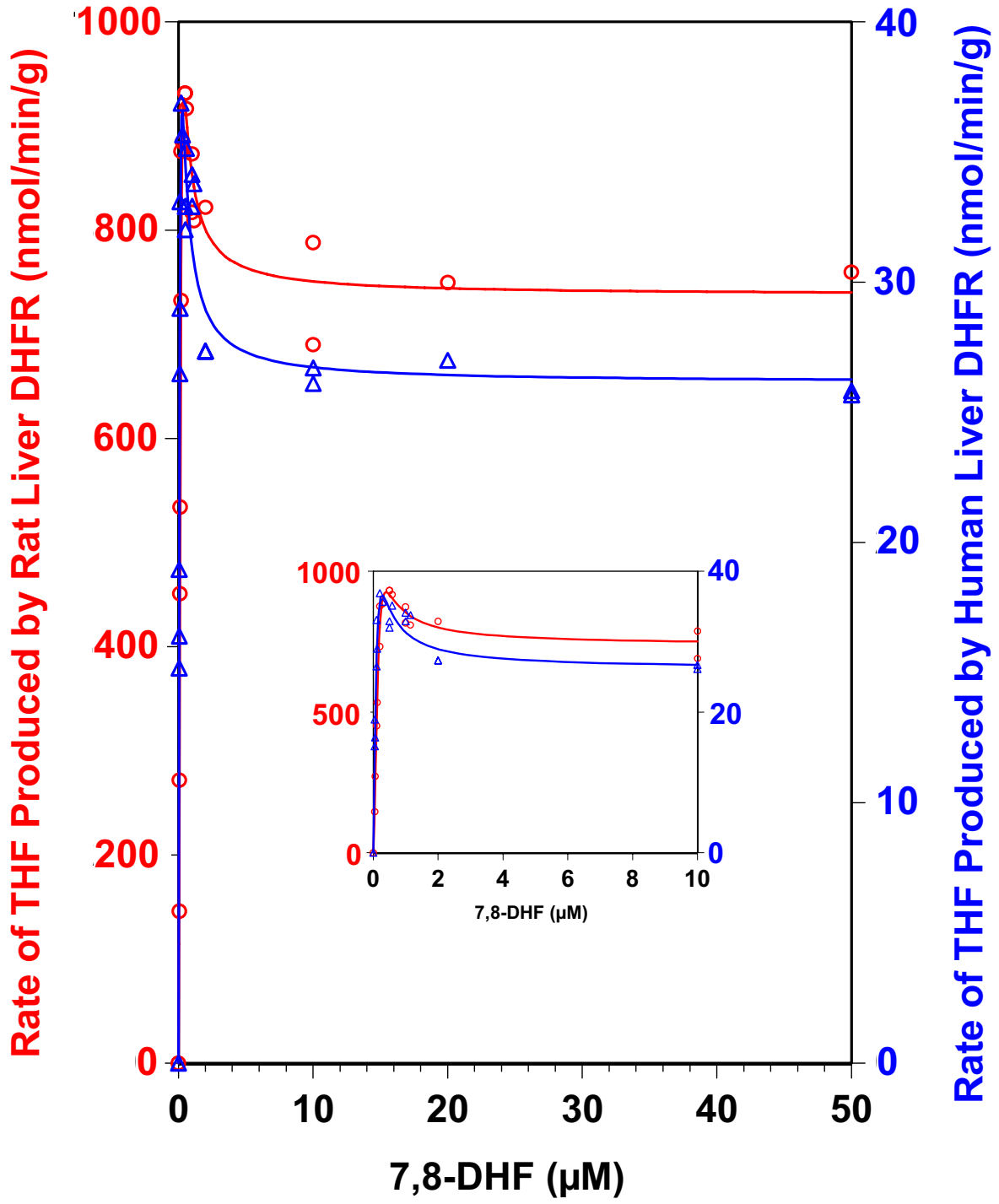


**Fig. S5.** $K_m$ for 7,8-DHF with human and rat liver DHFR. The $K_m$ for human DHFR (△) was measured with a liver fraction from subject 5, and for rat DHFR (O) with liver extract from CD rat #2.